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N- and C-Terminal Isoforms of Arg Quantified by Real-Time PCR Are Specifically Expressed in Human Normal and Neoplastic Cells, in Neoplastic Cell Lines, and in HL-60 Cell Differentiation

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The human ABL2 (or ARG) gene codes for a nonreceptor tyrosine kinase is involved in translocation with the ETV6 gene in human leukemia and has an altered expression in several human carcinomas. Two isoforms of Arg with different N-termini (1A and 1B) have been described. The C-terminal domain of Arg contains two F-actin-binding sequences that perform a number of actions related to cell morphology and motility by interacting with actin filaments. We have identified different-sized specific cDNAs in hematopoietic, epithelial, nervous, and fibroblastic cells by means of the reverse transcription (RT)-polymerase chain reaction (PCR) analysis of human Arg mRNA. Some of these cDNAs showed an adjunctive alternative splice event involving the 63 bp sequence of exon II, thus leading to four cDNA types with different N-termini: 1A long and short, and 1B long and short. Other cDNAs lacked a 309 bp sequence in the last exon involving one of the C-terminal F-actin binding domains, thus giving rise to two cDNA types: C-termini long and short. Quantified by real-time PCR—quantitative RT-PCR—these Arg transcript isoforms have specific expression patterns not only in different normal and tumor cell types, but also during cell differentiation and growth arrest. These isoforms maintained the open reading frames, and eight putative proteins were predicted. The different C-termini isoforms seem to retain the same quantitative reciprocal ratio of their respective transcripts. The Arg protein isoforms with different C-terminal actin-binding domains and different N-termini might have specific cellular localizations/concentrations, and differently regulated catalytic activity with different implications in normal and neoplastic cells.

Key words: Arg tyrosine kinase; mRNA splicing; transcript expression; protein expression; actin-binding sequence

INTRODUCTION

The Abelson family of nonreceptor tyrosine protein kinases is defined by products of human and mouse ABL2 (also known as ARG, Abelson Related Gene) and ABL1 genes, and Drosophila and nematode ABL genes [1–3]. In human acute leukemia, ABL2(ARG) gene can be involved in translocations with the ETV6 gene and produce different chimeric proteins [4–6]. An altered expression of Arg transcripts has been described in different tumors [7–10]. The human Arg protein has a high degree of amino acid sequence identity (90%–94%) with c-Abl in the tyrosine kinase SH2 and SH3 domains [3]. Two isoforms of both human Arg and c-Abl have been described as having different N-termini called 1A and 1B [3,11]. Four mouse c-Abl isoforms have been cloned with different 5′-ends arising as a result of the addition of alternative 5′-exons [12]. Although the long C-terminal domain of Arg is quite different from that of c-Abl, both contain three proline-rich sequences that bind to the SH3 domains of adaptor proteins [13,14]; c-Abl contains only one F-actin-binding sequence, while Arg contains two plus one microtubule-binding sequences [1,15]. The Arg product is located in the cytoplasm [16] whereas the c-Abl one is also nuclear.

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; 1AL, 1AS, 1BL, 1BS, long and short isoforms of the 5′-end (N-termini) type 1A and 1B of Arg transcript or protein; ATRA, *All-trans* retinoic acid; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; GM-CSF, granulocyte-macrophage colony stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTL, CTS, long and short isoforms of the 3′-end (C-termini) of Arg transcript or protein.

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The functional role of Arg is currently under investigation. Through its interactions with actin filaments, it performs redundant actions with c-Abl, playing a role in neurulation [17]. It has a function in adhesion-dependent neuritogenesis [18], and in synaptic structure and function [19,20]. Arg seems to be required for bacterial pathogenesis [21], and to be involved with c-abl in oxidative stress response [22] by regulating catalase activity [23]. The suppression of Arg kinase activity by STI571 induces cell cycle arrest [24], and it appears that Arg plays a role in homologous recombination DNA repair [25]. Lymphopenia occurs during the development of mice harboring a homozygous disruption of c-Abl [26], thus indicating that Arg is unable to substitute c-Abl functions in lymphoid tissues. Arg is ubiquitous with greatest expression in nervous tissues [27]. Arg mRNA increases during granulocytic and macrophage-like differentiation of HL-60 cells [28], and its expression is higher in mature than in immature B lymphoid cell lines [29].

During reverse transcriptase (RT)-polymerase chain reaction (PCR) analyses of human Arg mRNA, we had identified specific cDNAs of different sizes. These showed an adjunctive splice event immediately downstream of both the alternatively spliced 1A and 1B exons, and the lack of a sequence in the last exon coding the C-termini. These events gave rise to four cDNA types diverging at the 5′-end and two cDNA types diverging in the 3′-region. Their open reading frames were maintained, and the possible combinations of the different splicing events made it possible to predict eight putative proteins. There was a differential expression of the Arg transcript isoforms quantified by real-time PCR—quantitative RT-PCR—under diverse physiological conditions and in normal and tumor cells.

MATERIALS AND METHODS

Cells, Tissues, and Human Cell Lines

Unpooled samples of lymphocytes, monocytes and granulocytes were obtained from volunteer donors by means of density gradient separation in Ficoll-Hypaque and Percoll as described [30,31]. Purity (>90%) was determined microscopically after May Grunwald Giemsa staining. The leukemic blast cells were obtained at diagnosis from bone marrow of patients affected by acute myelogenous leukemia and separated by sedimentation on Ficoll-Paque gradient as mononuclear fraction. The leukemic blasts were >90% of total cells. These were characterized as myeloid (M1), monocytic (M5) blasts according to FAB classification [32]. Tumor tissue specimens (renal clear cell carcinoma, Grade 2; colon carcinoma, Dukes histopatological stage A, Grade 1) and corresponding normal tissues (renal cortex; colon mucosa) were obtained from patients soon after surgical treatment; fresh tissue fragments were immediately put into RNAlater (Ambion, Austin, TX) and frozen down in liquid nitrogen.

The human cell lines used (Table 1) were cultured with RPMI 1640 medium supplemented with 10% fetal calf serum, and tested during exponential growth. The growth characteristics, the differentiation of HL-60 cells to granulocytes by means of 4-d treatment with 1μM all-*trans* retinoic acid (ATRA) and to macrophage-like cells by means of 2-d treatment with 10 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA), as well as the immunofluorescence analysis of membrane CD11b marker expression, were performed as previously described [28]. The GFD8 cell line was cultured in the presence or in the 4-d absence of 5 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) for which it displays growth dependence. Removal of growth factor for 4 d led to reversible growth arrest in the absence of differentiation [34,35]. The growth characteristics of GFD8 cells were assessed by daily count and expression of the CD11b differentiation marker [28]. The ATRA, TPA, and GM-CSF came from Sigma-Aldrich (St. Louis, MO).

RNA Extraction, cDNA Synthesis, and Qualitative RT-PCR Analysis

Total RNA was obtained by cell and tissue extraction with TRIZOL (Invitrogen, Carlsbad, CA)

Table 1. Characteristics of Human Cell Lines Utilized

B Lymphoid
LP-1 (myeloma, mature plasma cell phenotype)
Raji (lymphoma, mature B cell phenotype)
AllPO (acute leukemia, immature early pre B cell phenotype)
T Lymphoid
Jurkat (acute leukemia, mature post thymic phenotype)
Molt-4 (acute leukemia, immature thymocyte phenotype)
Myeloid
K562 (chronic leukemia, erythroid lineage phenotype)
HL-60 (acute leukemia, granulocytic lineage phenotype)
GFD8 (acute leukemia, granulocytic lineage phenotype)
U937 (histiocytic lymphoma, monocytic lineage phenotype)
Neuronal
A-172 (glioblastoma)
Lan-5 (neuroblastoma)
Epithelial
Caki-1 (renal cell carcinoma, clear cells)
Hela (cervix carcinoma)
Fibroblastic
Hel 299 (lung embrionic fibroblast)

The cell lines were from American Type Culture Collection, except LP-1 and Lan-5 that were from German Collection of Microorganism and Cell Cultures, and AllPO [33] and GFD8 [34] that were a kind gift from A. Biondi (Milano-Bicocca University, Monza, Italy).

according to the manufacturer's instruction; it was spectrophotometrically quantified and its integrity was analyzed by electrophoresis in 1% agarose gel. The DNAse treatment of total RNA and the reverse transcription of an 8 μg aliquot of DNA-free RNA in a 40 μl-reaction-in-the-presence-of 0.5-μg-of-random examers was performed as previously described [28]; 2.5 μl cDNA was amplified in the presence of 0.4 μM primers, 2 mM $MgCl_2$, 0.2 μM dNTP, 2.5 U Taq Gold polymerase and 1x manufacturer's buffer (Applied Biosystem, Foster City, CA). The primers used in the combinations described in Figures 1 and 2 had the following sequences [3] and localizations:

41N 5′-ACACAGGTCCATGGTACC-3′ reverse (exon IV)
42N 5′-GCAGAGATCAGGACACTT-3′ sense (exon 1A)
132N 5′-AAGCTCCGGGGCTCCAGC-3′ sense (exon 1B)
112N 5′-CACCAGGGATAGGAAGGGG-3′ sense (exon XII)
113N 5′-GGGAAGGGTCATTGCCATC-3′ reverse (exon XII)
114N 5′-CTGCTCTGGAAGCCccgtg-3′ reverse (exon XII)
115N 5′-ACCAGATTCGCCTCTTGCTG-3′ reverse (exon XII)

41N, 42N, 132N primers have an additional 5′ eight-nucleotide tail containing the *Eco*RI restriction site. The capital and lower case letters of primer 114N show the fusion point of the sequences that juxtapose after the loss of a 309 bp fragment in the 3′-end of Arg cDNA. The amplification program was 95°C/10 min, (94°C/30 s, 60°C/30 s, 72°C/30 s) × 40 cycles, and 72°C/10 min. All the amplified cDNA were sequenced with the ABI Prism Kit Big Dye Terminator v3.0 sequencing kit, and the ABI Prism 3100 Avant Genetic Analyzer. The intron-exon junction of ABL2(ARG) (Figure 1) was determined with the NCBI Genome Map Viewer *Homo sapiens* database, Build 34,Version 1 (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606&query=arg).

Quantitative Real-Time PCR Analysis

Real-time PCR with TaqMan chemistry was used to quantify specific cell mRNA. The amplification was performed in an ABI PRISM 7900HT Sequence Detector. One microliter of the RT reaction (corre-

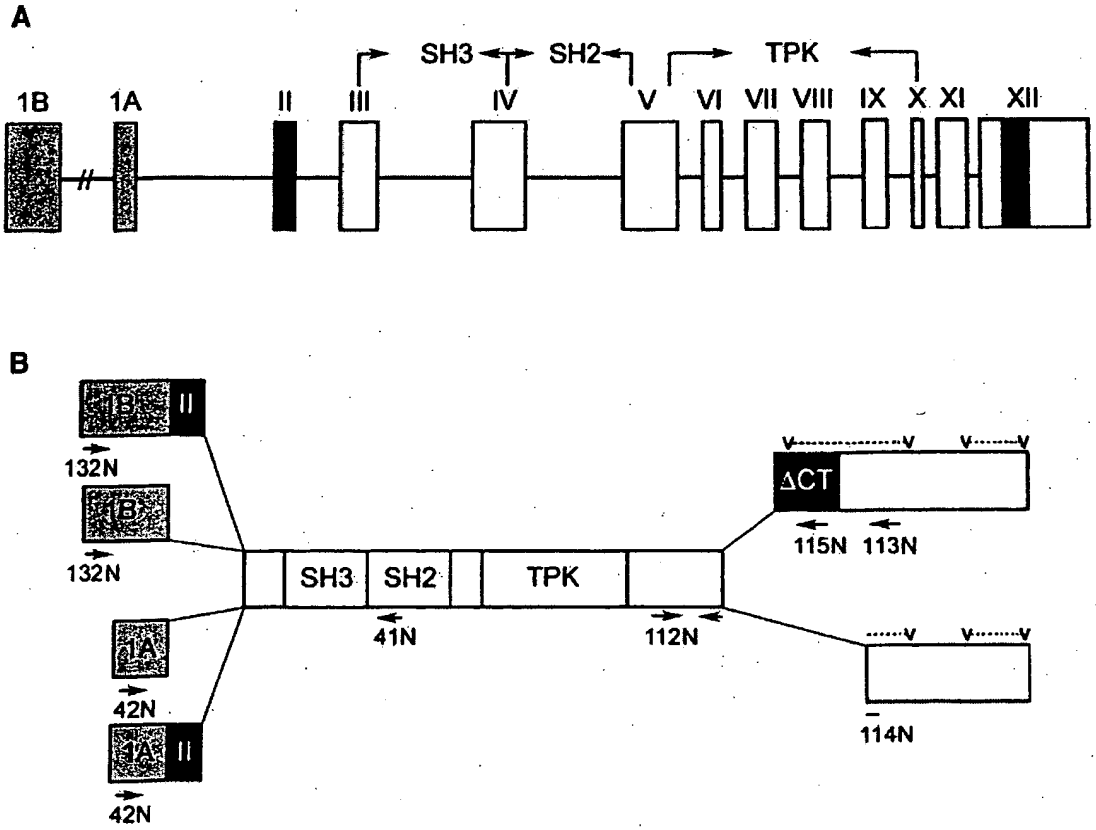

Figure 1. Schematic representation of the structure and cDNA coding sequences of the ABL2(ARG) gene. (A) The intron-exon structure of the ABL2(ARG) gene derived from the NCBI Genome Map View *Homo sapiens* database, Build 34, Version 1 and our own data. (B) Proposed isoforms of Arg proteins as predicted by the sequence of Arg cDNA obtained by RT-PCR analysis. The first alternative exons 1A and B are shaded, and the alternative spliced exon II (II) and C-terminal region (ΔCT) lacking in the CTS form are shown in black. SH3-SH2 and TPK indicate the SH3, SH2, and tyrosine protein kinase domains. The sense and reverse primers used in the RT-PCR are indicated with arrows. Primer 114N spans noncontiguous sequences. The position of the two F-actin-binding regions in the C-terminal domain are also indicated (v...v). The diagrams are not to scale.

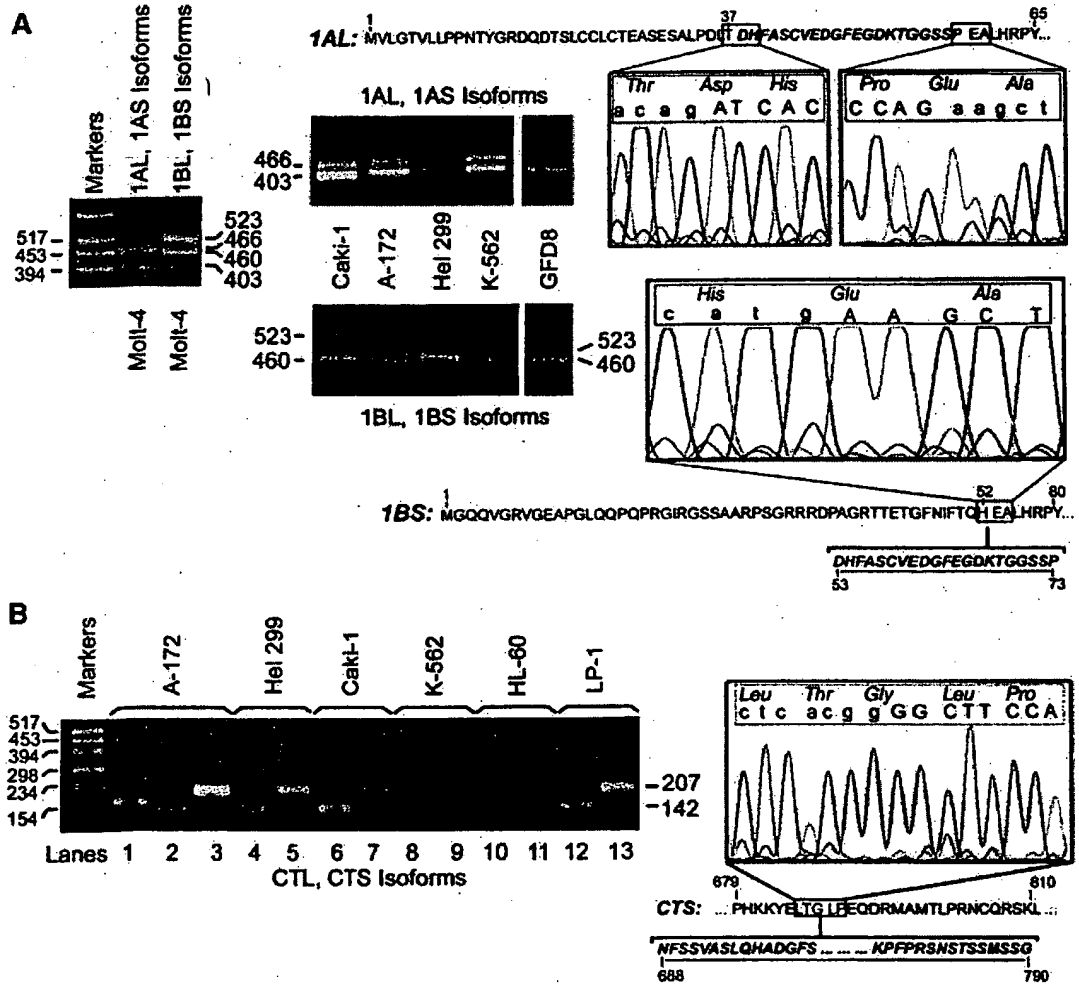

Figure 2. Detection of different ARG cDNA isoforms in various cell types by means of qualitative RT-PCR analysis. (A) 5'-end ARG isoforms. Left: The 41N/42N primer pair (see Figure 1) revealed two bands of 466 and 403 bp, corresponding to the 1AL and 1AS transcript isoforms, and the 41N/132N primer pair amplified two bands of 523 and 460 bp, corresponding to the 1BL and 1BS transcript isoforms. Right: Amino acid sequences of the 1AL (top) and 1BS isoform (bottom). The in-frame nucleotide sequence at the splicing sites (denoted by capital and lower case letters) are given. The 21 amino acids of exon II present in the 1AL form are indicated in bold. (B) 3'-end ARG isoforms. Left: The use of 112N/113N primer pairs (see Figure 1) led to obtaining two bands of 475 and 166 bp in A172 cell lines (lane 1). In these cells and other cell types, mRNA amplification with the 112N/114N (even lanes) and 112N/115N primer pairs (odd lanes from lane 3), respectively, evidenced a 142 and a 207 bp specific band, corresponding to the CTS and CTL isoforms. Right: Amino acid sequence of the CTS isoform and the in-frame site of the nucleotide sequences that juxtapose (lower case and capital letters) after the loss of the 309 bp fragment. The 21 amino acid sequences lacking in the 1BS isoform and the 103 amino acid sequences lacking in the CTS isoforms are underlined; the reported amino acid numbers are those of the entire coding sequence.

sponding to 200 ng of cDNA) was amplified in a 50 µl PCR mixture, containing 1× Universal PCR master mix (Applied Biosystems) and different concentrations of primers and probes (Table 2) whose sequences were selected with Primer Express 2.0 software (Applied Biosystems). The transcript of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene [36] was amplified as an endogenous control of RNA quality. Each cell line underwent at least two independent experiments, with each sample being analyzed in triplicate. The real-time PCR conditions were 50°C/2 min (for optimal AmpErase uracil-N-glycosylase [UNG] activity), 95°C/10 min followed by (95°C/15 s and 60°C/1 min) × 40 cycles. The ABI7900 system software raised the threshold cycle (C_T) values representing the cycle numbers needed to reveal the minimal amount of amplified material of a target transcript. The relative levels of the total Arg transcript in each sample were calculated with the averaged C_T values

Table 2. Sequences, Exon Localizations, and Concentrations of Primers and Probes Used for Transcript Quantification by Real-Time PCR and Their Respective Amplicon Lengths

Forms	Primer sense	Exon	nM	Primer reverse	Exon	nM	Probe[b]	Exon	nM	Amplicon (bp)
Arg Tot	(O) 5'AGTTTAGCACCAGggttcatcag3'[a]	XI- XII	300	(P) 5'CTTCCTATCCCTGGTGAAGCAT3'	XII	300	(y) 5'CACAGGCCTCTAGTGGATCCCCAGC3' (sense)	XII	200	135
Arg 1AL	(C) 5'TCTGCTCTACCCGACTTAACAGatc3'	1A-II	900	(B) 5'aaagctcCTGGACTACTGCCTCC3'	II- III	900	(k) 5'AGCTGTGTGGAGGATGGATTTGAGGGA3' (sense)	II	150	92
Arg 1AS	(F) 5'TGCTCTACCCGACTTAACAGaagc3'	1A- III	300	(E) 5'ACCTGATAGCCTCATTTAGTGCCT3'	III	300	(j) 5'ATCGTCCCTATGGTTGTGATGTTGAACCC3' (sense)	III	150	83
Arg 1BL	(A) 5'AATATCTTCACCCAGCATGatcac3'	1B- II	300	(B) 5'aaagctcCTGGACTACTGCCTCC3'	II- III	900	(k) 5'AGCTGTGTGGAGGATGGATTTGAGGGA3' (sense)	II	150	89
Arg 1BS	(D) 5'CAATATCTTCACCCAGCATGaagg3'	1B- III	300	(E) 5'ACCTGATAGCCTCATTTAGTGCCT3'	III	900	(j) 5'ATCGTCCCTATGGTTGTGATGTTGAACCC3' (sense)	III	150	82
Arg CTL	(S) 5'CCTTCCGAGAAATGGAGAATCA3'	XII	300	(T) 5'GCATGCTGTAGAGAAGCAACAGA3'	XII	300	(2) 5'CCCCATAAGAAATACGAACTCACGGGTAACTTCT3' (sense)	XII	200	82
Arg CTS	(Q) 5'TGGAGAATCAGCCCCATAAGA3'	XII	300	(R) 5'TCCTGGGAAGGGTCATTGC3'	XII	300	(v) 5'ATACGAACTCACGGgcttccagag3' (sense)	XII	200	78
GAPDH	(L) 5' GAAGGTGAAGGTCGGAGTC3'	II	200	(M) 5'GAAGATGGTGATGGGATTTC3'	IV	200	(x) 5'CAAGCTTCCCGTTCTCAGCC3' (reverse)	IV	100	226

[a]The capital and lower case letters show the sequences located on different exons and, in the case of probe (v), the sequences that juxtapose after the loss of 309 bp fragment in exon XII of Arg.
[b]The TaqMan probes were labeled at the 5'-end with the reporter dye molecule FAM (6-carboxy-fluorescin) (Arg probes) or VIC (GAPDH probe), and at the 3'-end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine).

of each sample [37]. Briefly, the averaged C_T value of the GAPDH transcript was subtracted from the averaged C_T value of the total Arg transcript of a specific cell type in order to obtain the Arg ΔC_T value. The difference ($\Delta\Delta C_T$) between the Arg ΔC_T values in a specific cell type and the Arg ΔC_T value of the LP1 cell line used as a calibrator was determined and expressed as $2^{-\Delta\Delta C_T}$, and represented the fold of Arg expression in relationship to the calibrator. The LP1 cell line was chosen as calibrator from the beginning of the study due to the more mature phenotype among the lymphoid cell lines studied [29]. The relative amount of Arg isoforms was calculated as $2^{-\Delta C'_T}$ [37]. A $\Delta C'_T$ was obtained by subtracting the averaged C_T value of total Arg from that of the target isoform, and was then transformed into $2^{-\Delta C'_T}$. This value represents the amount of a single isoform with respect to the total quantity of Arg transcript and is expressed as a percentage. The amplification efficiencies for total Arg, each Arg isoform, and the GAPDH transcripts were determined according to the validation experiments suggested by Applied Biosystems (User Bulletin No. 2) and were approximately equal. In order to confirm the specificity of the PCR reaction, the products of the real-time PCR were electrophoresed on a 1.2% agarose gel.

Western Blotting

The cells were lysed with 1% Triton X-100, 10 mM Tris-HCl PH 7.4, 150 mM NaCl, and the Protease Inhibitor Cocktail (Roche, Mannheim, Germany) as recommended by the manufacturer. The protein concentration was determined by means of a Bio-Rad microassay (Hercules, CA). The lysates (80 μg) separated in 7.5% polyacrylamide gel electrophoresis were blotted onto nitrocellulose membranes, and stained with Ponceu S in order to show equal lane loading. Western blotting was performed with rabbit polyclonal anti-Arg antibodies [17] directed against the SH2 and SH3 domains (a kind gift of A. Koleske, Yale University, CT). Anti-actin antibodies (Sigma-Aldrich) were used to detect β-actin protein. The detection was performed with secondary antibodies coupled to horseradish peroxidase and a SuperSignal Detection System (Pierce, Rockford, IL).

RESULTS

RT-PCR Qualitative Analysis of Arg Transcripts

The RNA extracted from several cell lines of hematopoietic, epithelial, nervous, and connective origin was analyzed by RT-PCR with two different sets of primers: 41N/42N and 41N/132N. The 41N reverse primer was located on the common exon IV of Arg, the 42N and 132N sense primers were, respectively, located on exons 1A and 1B. Both sets of primers 41N/42N and 41N/132N amplified two

bands of different sizes (respectively 466bp/403bp and 523bp/460bp), demonstrating that the ABL2(ARG) gene is normally expressed in the cells as four different 5'-end transcript isoforms, here called 1A long and short (1AL, 1AS) and 1B long and short (1BL, 1BS) (Figure 2). This was also confirmed with primers spanning different exons and specific for the individual isoform (not shown). These PCR products were all sequenced. The nucleotide sequence showed that 63 bp, which code for 21 amino acids are alternatively juxtaposed to the 1B and 1A first exon. The alternative splicing of the sequence maintain the open reading frame (Figure 2). On the basis of the intron-exon junction of Arg (derived from the NCBI Genome database), the 63 bp sequence was flanked by the consensus sequences of the acceptor (AG) and donor (GT) splice sites. Only the 1AS and 1BL forms were described during the first cloning of Arg cDNA [3] but, in the t(1;12) translocation present in leukemic patients, an identical 63 bp sequence had been found alternatively fused to the Arg common exons in the rearranged transcript [24–26]. However, the lack of 1A and 1B first exons in these rearranged transcripts made it impossible to identify whether this additional splicing event involved both the A and B forms of Arg.

On the basis of the information [17] that the mouse brain Arg sequence specifically excludes an exon encoding amino acids 688 (G) to 791 (S), we used PCR to test the same region of human Arg cDNA. Amplification of the cDNA obtained from the A172 glioblastoma cell line with the 112N/113N primer pair revealed two specific bands of 475 and 166 bp (Figure 2). The presence of different 3'-end forms in cellular RNA samples derived from different cell types was also demonstrated with the 112N/114N and 112N/115N primer pairs that amplified specific bands of 142 and 207 bp, respectively (Figure 2). The isoforms were called C-termini long (CTL) and short (CTS). The sequence revealed that the shorter band was the result of the in-frame loss of a 309 bp fragment encoding amino acids 688 (N) to 790 (G) in the C-termini. The lack of these 103 amino acids affects about half of the F-actin-binding domain [1,15] closest to the Arg kinase domain (Figure 1). The amplification of genomic DNA with the 112N/115N primer pair revealed a single band of 207 bp (as expected from the cDNA sequence), the 112N/114N primers did not reveal any amplified band (not shown). Primer 114N spanned the cDNA sequences that were juxtaposed after the loss of the 309 bp fragment which, in Arg cDNA [3], was delimited by the GGG sequence at both extremities. This GGG sequence also flanks the 5'-end of the 1B exon. On the basis of these data and the NCBI Genome Map Viewer *Homo sapiens* database, Build 34, Version 1, we derived the schema of the intron-exon order and the predicted putative protein isoforms of Arg (Figure 1).

Real-Time PCR Quantitative Analysis of Arg Transcripts

Total Arg transcripts

Real-time PCR with O/P primer pairs and the *y* probe complementary to common exons (Table 2) confirmed that the total Arg transcript was more abundant in mature (LP1, Raji, Jurkat) than in immature (ALLP0, Molt-4) cells of the lymphoid leukemic cell lines (Figure 3), as we previously showed with competitive PCR [29]. We also confirmed semiquantitative PCR results showing increased Arg transcript expression in the HL-60 myeloid leukemia cell line, which differentiated toward granulocytes or macrophage-like cells [28]. Among the cell lines tested, Arg transcript expression was highest in the A172 glioblastoma cells, in which c-Abl protein was not expressed because of the loss of functionally active germline ABL alleles [38].

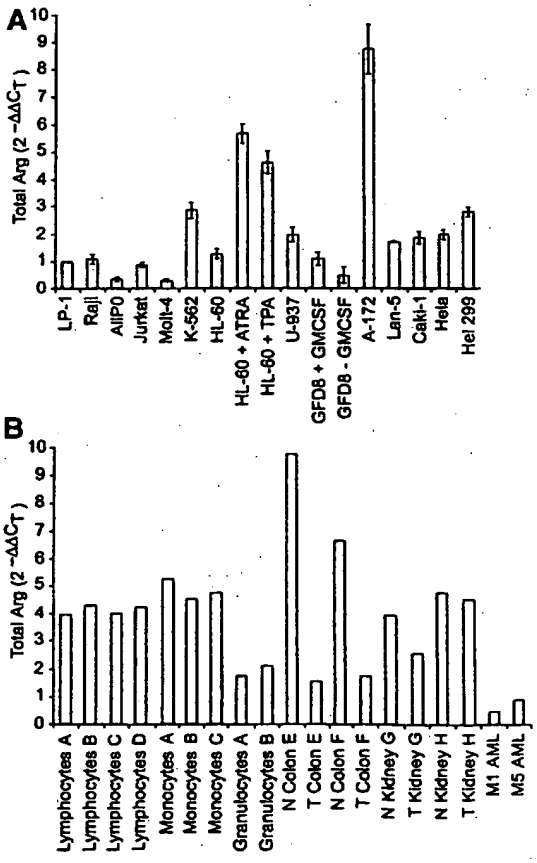

Figure 3. Relative levels of the total Arg transcripts in different human cell types as evaluated by Real-time PCR. The values expressed as $2^{-\Delta\Delta C_T}$ [37] represent the fold of Arg expression in each cell type respect to the LP-1 calibrator cell line, considered as having a value of 1. (A) Cell lines. Mean values of two independent experiments performed in triplicate; the vertical bars indicate the range of variability. (B) Cells and tissues from single individuals denoted by the letters A through H. N, normal; T, tumor; M1, M5, acute myelogenous leukemia FAB types.

The mature lymphocytes, monocytes, and granulocytes from the single normal donors had not only a higher Arg transcript expression than the tumor cell lines of the lymphocytic (LP1, Raji, ALLP0, Molt-4, Jurkat), monocytic (U937), and granulocytic (HL-60, GFD8) lineage, but also than the blasts of myelocytic (M1) and monocytic (M5) spontaneous acute leukemia. A higher expression of Arg had also been observed in normal colon mucosa and in one renal cortex than in the respective carcinomas. On the whole, it seemed that in tumor cells, there was a downexpression of Arg with respect to the normal cells.

5′-End isoforms

The differential quantification of each 5′-end isoform with specific sets of primers and probes (Table 2) demonstrated that the four Arg isoforms were present in different amounts (Figure 4A and B) and had a characteristic expression pattern in the diverse cell types. In hematopoietic cell lines, in Lan-5 neuroblastoma cell line, in lymphocytes, monocytes, and granulocytes, in the M1 and M5 leukemic blasts, the prevailing form was the 1BL followed by its short counterpart (1BS). In these cells, the 1AL form might be quantifiable, while the 1AS form (the only 1A form so far described in literature [3]) was in general the least represented. The most expressed form in A172 glioblastoma and Hel-299 fibroblastic cell lines was 1AL followed by 1AS, although the B forms were quantifiable. In Caki-1 and Hela epithelial cell lines, the 1AL form was the most abundant, but all the other forms (1AS, 1BL and 1BS) were consistently represented accounting for 15%–25% of the total Arg level. A relative distribution of the forms in favor of 1A was found in the two normal renal tissues, this pattern changed with a redistribution of the forms and the prevalence of 1BL in the two renal carcinoma. The forms more represented in the two normal colon mucosa were 1BL and 1BS, this also being true for the tumoral counterpart.

3′-End isoforms

Differential quantification of the 3′-end isoforms showed that all the tested cells contained both CTS and CTL (Figure 4C and D). CTS was prevalent in B lymphoid cell lines, but there was a decrease in CTS with a concurrent increase in CTL in the neoplastic LP1 (plasmacells), Raji (mature B cells), and AllPO (early pre B cells) cell lines that reflected the difference in maturation. CTS was also greater than CTL in K562 myeloid and Hel-299 fibroblast cells. In the Caki-1 and Hela epithelial cell lines, CTS was more abundant, but the level of CTL was approximately similar. CTL was prevalent in Jurkat and Molt-4 T cell lines, in U937 monocytic cell lines, in A172 glioblastoma, and Lan-5 neuroblastoma cell lines. All donor lymphocytes (mainly T cells), monocytes, and granulocytes had a prevalence of the CTL form. The two forms were equally expressed in one case of normal colon mucosa, whereas CTL was slightly predominant in the other. In both cases of colon carcinoma, CTS was greater than CTL. The chief form in the two normal renal cortexes was represented by CTS. This pattern was unchanged in one of the renal carcinoma, but was inverted in the other.

Real-Time PCR Quantitative Analysis of the Arg Transcript Isoforms in Treated Cells

The HL-60 cells differentiated to granulocytes with 1 μM ATRA and to macrophage-like cells with 10 nM TPA. The ATRA-treated HL-60 cells stopped growing at d 4 (Figure 5A) showing granulocytic phenotype and morphology [28]. At d 2, the TPA-treated HL-60 cells stopped growing (Figure 5A) and about 60% were adherent to the flask, and were fully viable macrophage-like cells [28]. In the granulocytic differentiation of ATRA-treated HL-60 cells, the expression pattern of the 5′-end isoforms did not change significantly, but a prevalence of the 3′-end CTL form (Figure 5B) as in the granulocytes of volunteer donors (Figure 4B) was observed. In the macrophage-like differentiation of TPA-treated HL-60 cells, the expression profile of the 5′-end isoforms changed dramatically, with a significant increase in the 1A forms and particularly of the 1AL. The 3′-end forms showed redistribution in percentage of CTS and CTL, but the increase in CTL was insufficient to make it more abundant than CTS (Figure 5B).

Given that in HL-60 cells, the differentiation is associated to growth arrest we also investigated the GFD8 cell line in which proliferation blocking could be dissociated from differentiation. The GFD8 cells share properties with early myeloid progenitor cells and are GM-CSF dependent for growth. The presence of GM-CSF does not change the cellular phenotype [34]. Removal of growth factor for 4 d leads to reversible growth arrest (Figure 5A) in the absence of differentiation and without a significant loss of viability [35]. GM-CSF deprivation led to a change in Arg expression at d 4, with the 1A, especially 1AL, forms becoming the most abundant. The 3′-end forms had an increase in the relative difference between CTS and CTL, with CTL remaining preponderant (Figure 5B).

Arg Protein Isoforms Evaluated by Western Blotting

Western blotting analysis of different cell lysates with anti-Arg antibodies revealed a set of bands as previously described [17,29]. The absence or presence of the 21 amino acids of exon II was not sufficient to reveal the different N-terminal isoforms by means of electrophoresis mobility on one-dimension polyacrylamide gel. Thus, the differently sized bands detected by anti-Arg antibodies probably reflected the different sizes of the Arg coding region

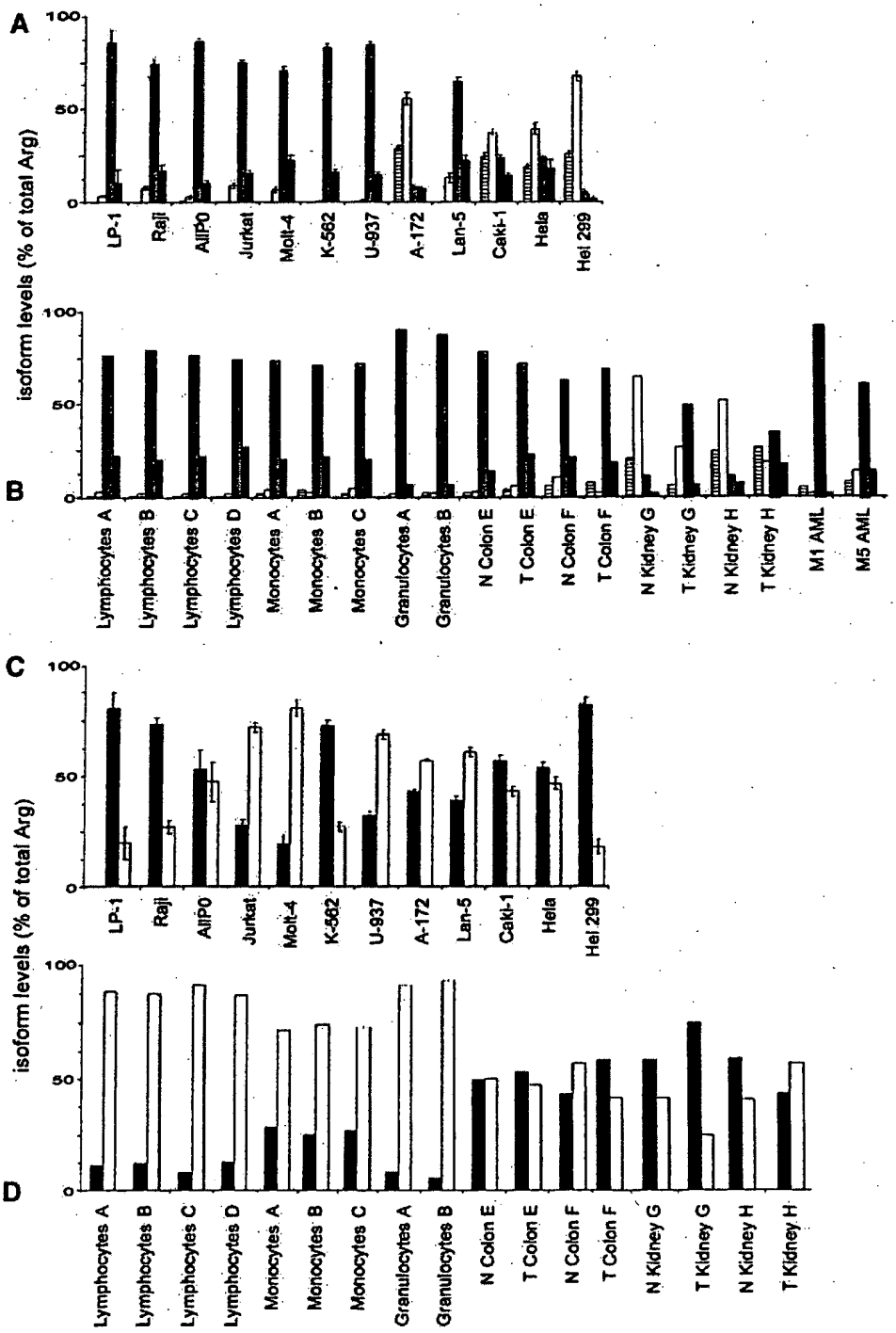

Figure 4. Relative levels of the different isoforms of the Arg transcripts in different human cell types. The values calculated as $2^{-\Delta C_T}$ [37] are reported as the percentage of the individual isoforms with respect to the total Arg transcripts. (A, B) 5'-end isoforms (≡ 1AS; □1AL; ▩1BL; ■1BS). (C, D) 3'-end isoforms (■CTS; □CTL). (A, C) Cell lines. Mean values of two independent experiments performed in triplicate; the vertical bars indicate the range of variability. (B, D) Cells and tissues from single individuals denoted by the letters A through H. N, normal; T, tumor; M1, M5, acute myelogenous leukemia FAB types.

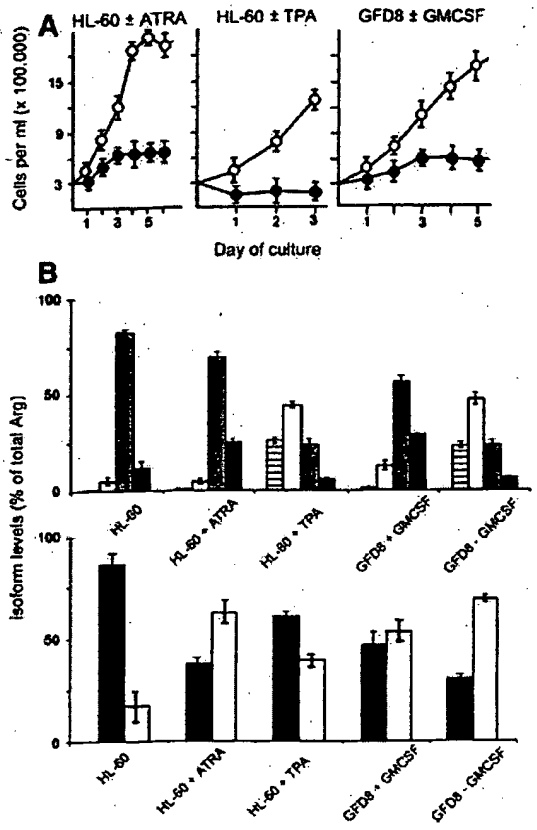

Figure 5. Relative levels of the different isoforms of Arg transcripts in treated cell lines. (A) Growth rate of HL-60 cells, untreated (○) and treated (●) with ATRA or TPA, and GFD8 cells cultivated in the presence (○) or absence (●) of GM-CSF. Exponentially growing cells were plated in 50 mL of medium at a density of 3×10^5/mL. The total cell number was determined in a Thoma chamber. In the case of the TPA-treated cells, the data refer to the adherent cells detached from the flask after incubation at 37°C with trypsin, and the counted cells are expressed as the number of cells/ml of the initial 50 mL volume. Mean values ± SD of three independent experiments. (B) HL-60 cells untreated and treated with 1μM ATRA for 4 d or 10 nM TPA for 2 d. GFD8 cells cultivated in the presence or absence of 5 ng/ml GM-CSF for 4 d. Top: 5'-end isoforms (▭1AS; □1AL; ▩1BL; ■1BS). Bottom: 3'-end isoforms (■CTS; □CTL). The values calculated as $2^{-\Delta C_T}$ [37] are reported as the percentage of single isoforms with respect to the total Arg transcripts. Mean values of two independent experiments performed in triplicate; the vertical bars indicate the range of variability.

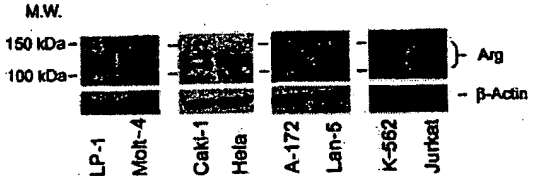

Figure 6. Western blot analysis of 80 μg of cell lysate from different cell lines separated on 7.5% polyacrylamide gel electrophoresis with polyclonal anti-Arg and anti-β-actin antibodies. The different panels are different blots and the exposure time ranged from 30 to 60 s.

caused by the loss of 103 aminoacids in the C-termini (Figure 6). The protein bands revealed in the different cell types also had a different reciprocal intensity, with a general agreement between band intensity and the reciprocal amount of CTL and CTS transcripts in the different cell lines as quantified by real-time PCR. The shorter protein was greater in K562 cells, and the longer protein in Molt-4 and Lan-5 cells, whereas both proteins were roughly equivalent in the Caki-1 and Hela cells. Instead, in the LP-1 cells the most abundant band was that corresponding to the longer protein, which disagreed with the transcript data. Finally, it was worth noting that the longer protein seen in A172 cells ran faster than those seen in the Lan-5 and in the other cells. Moreover, the total amount of Arg proteins did not correlate with the Arg mRNA in the A172 cells (Figure 3).

DISCUSSION

During our PCR analysis of the 5'-end of human Arg cDNA, we found two differently sized amplified fragments (long and short) for both 1A and 1B forms. These 1AL, 1AS, 1BL, and 1BS cDNAs diverged in positions that were unique for the long and short forms, thus suggesting that they arose as a result of the combination of two alternative splicing events, one involving the 1A and 1B exons, and the other a second exon. The fact that a 63 bp fragment (which is flanked by a splice acceptor and donor site in the genome) can be alternatively juxtaposed to exons 1A and 1B of Arg suggests that this sequence is the real exon II. In human Arg, the 1A exon therefore ends at amino acid 37 (T) and the 1B exon at amino acid 52 (H), both of which can or cannot be followed by the 21 amino acids of exon II. The long Arg forms (1AL or 1BL) containing exon II are always the most abundant in the cells studied, which suggests that this exon plays an important role in the function of both the A and B forms of Arg proteins, although the exact function of the 21 amino acid sequence has not yet been established. Our findings were also supported by the fact that the translocation involving the ABL2(ARG) gene in leukemia produces the ETV6/ARG fused proteins containing (long form) or lacking (short form) exon II [4–6]. It has been shown that the presence of exon II in the ETV6/ARG long protein leads to more pronounced oncogenic activity [39] in comparison with the short form. The presence of this Arg exon II may therefore be important and also reflect the situation normally existing in the fusion proteins of c-Abl (ETV6/ABL; BCR/ABL), in which the fusion point comes soon after the end of the first exon of c-Abl, but whose second exon does not have any analogy with this second exon of Arg. Furthermore, the N-terminal domains of Arg and c-Abl seem to play an important role in regulating their catalytic activity [40,41], and therefore any study of the kinase activity of Arg

should consider the unique presence of Arg exon II, keeping it distinct from exons 1A and 1B.

Our real-time PCR data showed that the 5'-end short and long forms of Arg mRNA are consistently represented in cells, and four different N-terminal proteins can be predicted from the Arg cDNA sequence even though the difference in N-terminal amino acids is too small to allow their separation by means of one-dimensional gel electrophoresis. The 5'-end isoforms have an expression pattern which differs in the diverse cell types and, in general, it seems that there is a specific prevalence of 1AL and 1AS or 1BL and 1BS isoforms in the various cells: this may suggest, for example, that the 1B first exon has a specific role in the hematopoietic cells in which the 1BL and 1BS isoforms prevail. Moreover, the specific function of the B forms may be differently modulated by the distribution of the long and short forms, which having distinct N-termini may differentially regulate catalytic activity [40,41]. The same can be said for the cells in which the A isoforms predominate.

All of the isoforms may therefore have an as yet unknown functional role, a hypothesis that is also supported by the changes observed during HL-60 cell differentiation and growth arrest of GFD8 cells, and by the fact that in addition to a prevalent form, the others are similarly abundant in some cell types (Caki-1, Hela). The functional impact of the various isoforms can of course be different, depending on their relative abundance. It is worth noting that four mRNA isoforms of mouse c-Abl have been described that diverge at the first exon [12]: the type I and IV isoforms are predominant and translated, and it has been suggested that these two proteins play different roles, with type I being involved in LPS-induced lymphoid differentiation and type IV in apoptosis [42].

The PCR-revealed 309 bp deletion in the last exon of Arg causes the loss of 103 amino acids from the C-termini that affect the actin-binding domain closest to the kinase domain. The two major protein bands revealed by Western blotting with anti-Arg antibodies may represent the two translated isoforms which, on the basis of their amino acid composition, differ by about 10 kDa. The expression pattern of the 3'-end transcript isoforms also varies in the different cell types, and their reciprocal ratio is maintained in the probably translated proteins. The presence of different Arg protein isoforms may have various implications. The C-terminal domains of Arg with diverse actin-binding domains might lead to different interactions with the actin cytoskeletal structure, and to distinct localizations/concentrations of the N-terminal isoforms that might be involved in the activation of different metabolic pathways [40]. A down regulation of Arg expression was observed in the tumors studied as reported in other tumors [7–10]. The Arg isoforms might also play a role in neoplasms in which an altered Arg expression can be accompanied by variation in the expression pattern of specific Arg isoforms, as we noted in a few tumor cases. Although these data need to be confirmed in a greater number of cases, they open the possibility that any variation of the expression pattern of Arg isoforms might have different and specific effects on morphology and motility or on other specific biological events in tumor cells.

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